

EXHIBIT J

TO DECLARATION OF SCOTT D. TANNER, PHD.

U.S. Patent Application Ser. No. 10/614,115

The potential for elemental analysis in biotechnology†

Vladimir I. Baranov, Zoë A. Quinn, Dmitry R. Bandura and Scott D. Tanner

MDS SCIEX, 71 Four Valley Drive, Concord, Ontario, Canada L4K 4V8

Received 11th February 2002, Accepted 15th May 2002

First published as an Advance Article on the web 18th June 2002

JAAS

FULL PAPER

www.rsc.org/jaas

The ICP-MS brings particular attributes to the method that distinguishes it from organic mass spectrometry: relative insensitivity to concomitant organic or metallic species, equivalent sensitivity to all similarly-tagged complexes, inherent ability to provide absolute quantitation and linearity of response. All these positive features fit the requirements of modern-day biotechnology. In this work, the potential of ICP-MS is discussed in the context of several urgent problems of biotechnology including: the determination of the state of phosphorylation of proteins, quality control of biologically active materials and control of efficiency of cell line transfections.

Introduction

Elemental analysis is quite successful for the quantitation of metal containing proteins.^{1–10} For a long time, it was accepted that if a biologically active molecule does not ligate or incorporate a metal atom, it should be analyzed by another means. For example, organic mass spectrometry (e.g., electrospray MS/MS) has been a method of choice for protein identification and phosphorylation site analysis. In spite of a limited set of “building blocks”, biologically active molecules exhibit an incredible variety of functionality, reactivity and structural complexity. This is especially true for proteins and peptides, which are (in their inconceivable diversity) still polymers of the same monomer molecules in different combinations making specific characterization of individual proteins difficult. In addition, there are numerous vital proteins present in cells in trace amounts. Therefore, these molecules represent a significant challenge for any analytical method including inductively coupled plasma mass spectrometry (ICP-MS) for which speciation capabilities are still very restricted.

Recent developments in ICP-MS have the potential to expand the “tool box” for protein analysis. One is related to dramatic improvements in the detection of phosphorus¹¹ and sulfur^{12–14} in biological samples, which enables the determination of the state of phosphorylation of proteins.^{15,16} A second development involves measurements of the atomic composition of a tag conjugated to a biologically active material,^{17–22} for example, an antibody molecule. The mass-to-charge ratio of an element contained in a tag provides the potential for multi-analyte detection using different elements and isotopes conjugated to different antibodies.

The quantitative determination of phosphorus in biological samples can yield important information about the state of phosphorylation of proteins. A preferred method of specific detection of phosphorus associated with biomolecules of interest is radioisotope (γ -³²P) labeling. This method has an obvious drawback—the necessity of radioisotope handling and disposal. Of all other methods used for detection of P and S, sector field ICP-MS is the most sensitive. Application of ICP-MS to ³¹P⁺ detection in the eluent of micro-LC was described recently¹¹ and simultaneous measurement of phosphorus and sulfur in proteins by ICP-MS was reported in refs. 15 and 16. Sector field ICP-MS was used at a medium resolution of 4000,¹⁵ with low solvent load on the plasma by use of micro-LC at 4 μ L min^{−1} yielding good detection limits

for P⁺ and S⁺. Due to isobaric interferences, a low resolution (quadrupole) analyzer should employ alternative (for example, chemical) means of separation. Products of oxidation of P⁺ and S⁺ with O₂ (PO⁺ and SO⁺, respectively) in a Dynamic Reaction CellTM were used as analyte ions allowing sub-ng mL^{−1} detection of P and S in aqueous samples.¹⁶ In this work, we intend to demonstrate the robustness of this method for total quantitation of proteins in samples containing a cell lysate.

Direct quantitation of naturally occurring P and S can be successfully complemented by measurement of an elemental tag that is specifically attached to a protein of interest to make this molecule more distinguishable from the background of similar molecules. Because tags especially designed for elemental analysis are not yet developed, we have used a variety of immuno-reagents that include metals for different purposes. Colloidal gold or extremely small gold clusters (less than 2 nm in diameter) are extensively used to visualize protein structure in the cell and to detect receptor–ligand binding by electron microscopy.^{23–27} Gold-containing tags are obviously convenient for elemental analysis and have dominated the first attempts to utilize this technique. Elemental nano-particles are especially attractive for quantitation because of their uniform size and significant number of atoms per conjugate. There is also the possibility of increasing the signal response yet further by using silver enhancement.²⁷ Elemental analysis (employing electrothermal atomic absorption spectrometry, ICP-MS) of colloidal gold and conjugated gold clusters was first reported for the determination and quantitation of low-density lipoprotein^{17–19} and in the context of an immunoassay with ICP-MS detection in refs. 20–22. We expect that the gold-cluster antibody conjugates can be successfully used also in multitarget assays. Simultaneous analysis requires several distinguishable tags, the choice of which is still limited, but nevertheless sufficient for a variety of applications.

Another class of metal containing tags are the releasable fluorescent probes^{28,29} which, for example, have been marketed by Wallace (AutoDELFIATM)³⁰ utilizing the fluorescent properties of four lanthanide chelates (Eu, Tb, Dy and Sm) to measure the concentrations of various antigens in an automated immunoassay system. Some of the Wallace AutoDELFIATM antibodies are labeled with 6–8 atoms of lanthanide. This feature is also advantageous for mass spectrometric determination of the element of the tag because it allows signal enhancement proportional to the number of atoms of the tag isotope. It is straightforward to quantify the lanthanide ions directly employing ICP-MS as has been successfully demonstrated in refs. 22 and 31.

Tungsten, silver, and platinum^{32–34} might also be considered

†Presented at the 2002 Winter Conference on Plasma Spectrochemistry, Scottsdale, AZ, USA, January 6–12, 2002.

as elemental tags but are not yet commercially available. EDTA-Cd²⁺ chelate was also used as a label in immunoassay based on graphite furnace atomic absorption spectrometry.³³

Due to its substantial analytical advantages (such as multi-element capability, high speed of analysis, exceptional limits of detection and wide dynamic range), elemental analysis is now being investigated for use in many areas of biotechnology including on fields of clinical assays, quantitation of trace proteins and signal transduction (proteomics). Selected aspects of this development are discussed in this paper.

Experimental

Experimental measurements were made on a commercial ICP-MS, either on ELAN 6100 or on ELAN DRC^{plus} (Perkin Elmer SCIEX). The sample uptake rate was adjusted depending on the particular experiment and sample size. A MicroFlow PFA-ST concentric nebulizer (Elemental Scientific, Inc) was used in all instances. Experiments were performed using an autosampler (Perkin Elmer AS 91) modified for operation with 96 deep well microtiter plates. All dilutions used de-ionized (Elix/Gradient water purification system, Millipore) water. Research purity (99.998%) oxygen (Matheson Gas Products, Whitby, Ontario, Canada) was used as reaction gas. Quantitation of phosphorus and sulfur were conducted in a class 1000 clean room, with the sample introduction system protected by a class 100 portable laminar flow bench. It should be noted that, although extreme precautions were taken to avoid contamination, the method of P and S determination described here also was used with similar results on a different instrument, with UHP grade (99.98% min.) oxygen (Matheson Gas Products, Whitby, Ontario, Canada) and not in the clean room, having only a Class 1000 laminar flow bench to protect the sample introduction system. Other reagents included: 1 × Phosphate Buffered Saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.4), 1% Bovine Serum Albumin (BSA), 10% (v/v) HCl (Seastar Chemicals Inc.), buffer A (20mM Tris, 90 mM NaCl, 2 mM CaCl₂, pH 7.5), NANOGOLD goat anti-human Fab' conjugate (Fab'-nanoAu; #2053, Nanoprobe), Ir and Ti diluted from stock 1000 ppm solutions (SPEX), 96 well microtiter plates. A high prep sephacryl column (XK 16, Amersham Pharmacia) and sephacryl S200 matrix (Amersham Pharmacia), were used for ICP-MS-linked gel filtration immunoassays.

Results

Determination of P and S

A method for the determination of the degree of phosphorylation of proteins in biological samples was developed recently¹⁶ and is based on the chemical resolution of ³¹P⁺ and ³²S⁺ from polyatomic ions by oxidation to ³¹P¹⁶O⁺ and ³²S¹⁶O⁺ in a reaction with oxygen gas supplied to a dynamic reaction cell (DRC). The oxide ions are then used as analyte ions. Both S and P are measured simultaneously under the same instrument operating conditions. This opened the possibility to use sulfur as an internal standard for phosphorus in the determination of protein phosphorylation.

For a reaction cell without axial field detection limits (3σ, 5 s integration) of 0.20 ng mL⁻¹ and 0.52 ng mL⁻¹ were achieved for P and S, respectively. As can be seen in Fig. 1, with an axial field, the background equivalent concentrations were equal to 0.63 ng mL⁻¹ (on ³¹P¹⁶O⁺) and 3.9 ng mL⁻¹ (on ³²S¹⁶O⁺) which translates into improved detection limits of 0.06 ng mL⁻¹ for P and 0.2 ng mL⁻¹ for S.

Based on this study it can be shown that the ratio of P/S measured as PO⁺/SO⁺ by the Dynamic Reaction Cell can be indicative of the total phosphorus content in an unpurified mixture of cell proteins. In this work, we intend to show that good correlation of the P/S ratio for similar cell cultures with

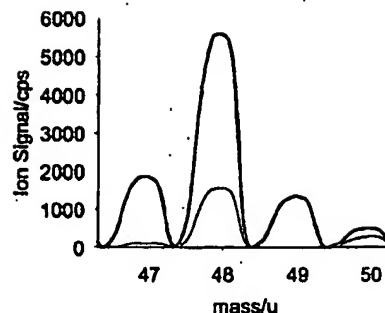


Fig. 1 Mass spectra for 10 ppb P, S measured as ³¹P¹⁶O⁺ and ³²S¹⁶O⁺. AFT = 200 V (axial field), O₂ = 0.5 sccm, *q* = 0.4, *a* = 0.02. Fine line: DIW and solid line: 10 ppb standard, both measured under the same experimental conditions. Basics of DRC operation and its optimization strategy and parameters are provided in refs. 36–39.

significantly different total protein concentration can be achieved. For unsynchronized cell cultures sampled in different growth phases one should expect little or no difference in the P/S ratio although the absolute phosphorus signals might fluctuate significantly.

Table I shows the P/S ratios for the samples of the lysates of the HEK 293 cell line (human embryonic kidney cells) prepared in different buffers and measured over the course of several days with different sample dilution factors under similar operating conditions. Each sample contained (before dilution) approximately ~10⁷ cells harvested from a single dish. Some cells were collected from dishes in TBS (Tris buffered saline) and homogenized using Tris-based lysis buffer (containing Tris, NaCl and NP-40). The cells were washed in dishes in TBS, then the buffer was fully aspirated, and concentrated HCl was added. The acidified protein mixture was collected into Eppendorf test tubes.

As one can see, despite significant variations in sample preparation and in absolute signals for ³¹PO⁺ and ³²SO⁺ measured for 17 different cell lysate samples incubated and prepared in 4 different groups of cell cultures on 4 different days, variation of the ratio of PO⁺/SO⁺ is small within the group. In addition, between groups variation was acceptable (10.5% RSD) taking into account different buffers, dilutions and cell growth state. Detection of a second sulfur isotope (³⁴SO⁺) gives additional verification of the presence of interferences during S measurement. If large variation in the ratio of signals at *m/z* = 48 and *m/z* = 50 occurs, possibility of contamination with Ti, Cr, V or Ca has to be considered. Although some of the possible contaminants react with O₂ and are suppressed, care must be taken in order to ensure lowest possible content of impurities in the buffers. We speculate that the P/S ratio is characteristic for a given cell culture and its growth conditions (including timing) and can be used for comparison of protein mixtures in lysates of different cell cultures where the variation in this ratio could be higher.

In order to demonstrate this hypothesis, P and S were measured for two different cell cultures. Two sets of samples were prepared. 14 tissue culture dishes (100 mm) with cell culture 1 (C1) and 6 dishes with cell culture 2 (C2) were washed with ice-cold TBS two times. The cell monolayer was collected by scraping cells off the dish into TBS. Cell pellets were collected by centrifugation at 500 g for 5 min. Cells from 7 dishes of C1 were resuspended in a glycerol-containing lysis buffer, and from 7 others in Tris-based lysis buffer. Similarly, cells of C2 from 3 dishes were resuspended in a glycerol-containing lysis buffer, and from the other 3 in a Tris-based lysis buffer. Cells were completely disrupted using a mechanical

Table 1 Results for repeated preparation and measurements of P and S content for HEK293 cell lysates

Dish number	PO ⁺	³² SO ⁺	³⁴ SO ⁺	³¹ PO ⁺ / ³² SO ⁺	³⁴ SO ⁺ / ³² SO ⁺
Tris-based lysis buffer, 1 : 100 dilution with DIW					
HEK293-1	23555	15659	947	1.504	0.060
HEK293-2	23914	15861	979	1.508	0.062
HEK293-3	22092	15297	899	1.444	0.059
HEK293-4	22797	14936	915	1.526	0.061
Concentrated HCl, 1 : 20 dilution with DIW					
HEK293-5	157023	117181	6865	1.340	0.059
HEK293-6	153103	113692	6691	1.347	0.059
HEK293-7	157488	115882	6799	1.359	0.059
HEK293-8	135977	102473	6032	1.327	0.059
Concentrated HCl, 1 : 50 dilution with DIW					
HEK293-9	78127	66756	3710	1.170	0.056
HEK293-10	87685	73855	4041	1.187	0.055
HEK293-11	71833	59602	3258	1.205	0.055
Concentrated HCl, 1 : 10 dilution with DIW					
HEK293-12	518003.6	416451	22738	1.244	0.055
HEK293-13	421511.4	366104	19923	1.151	0.054
HEK293-14	488301.1	424429	23074	1.150	0.054
HEK293-15	517139.2	431385	23334	1.199	0.054
HEK293-16	535357.8	445222	24172	1.202	0.054
HEK293-17	492968.4	432567	23571	1.140	0.054
			Average	1.294	0.057
			RSD (%)	10.5	4.8

homogenizer and checked under microscope for complete disruption.

Approximate expected concentrations of the samples were equal for each buffer: 5.6×10^7 cells ml^{-1} for C1 and 4×10^7 cells ml^{-1} for C2. When absolute phosphorus signal was compared between cell cultures (P_{C1} and P_{C2} for cell cultures C1 and C2, respectively), the ratio P_{C2}/P_{C1} was found to be 1.16 for Tris-based lysis buffer samples and 1.61 for glycerol-containing buffer. The difference was significantly larger than the errors of the measurement. When the signal for phosphorus was normalized to sulfur, the normalized ratios P_{C2}/P_{C1} were much closer (1.58 and 1.45, respectively) and within the errors of measurement consistently indicating difference between cell cultures C1 and C2.

It should be noted that the sulfur content depends on the protein composition. In addition, DNA has structural phosphorus implying that different cultures could be different not only by protein phosphorylation (for example, cells originated from different species). In our opinion, although probably not universal, this method can be in many cases quantitative. This may be especially so in two extremes: cell cultures and purified proteins. Generally, it might be used successfully if the number of sulfur-containing amino-acid residues is similar (or well known) between the samples. Because this method allows simultaneous quantitation of S and P in one assay, it should be more robust, faster and potentially more sensitive than other conventional methods.

ICP-MS linked gel-filtration chromatography

Purification and determination of the dissociation constant for antibody-antigen complex can be performed using the gel-filtration chromatography. This technique is useful in estimating K_d , the dissociation constant (reciprocal of the affinity constant) for the antibody-antigen complex. The binding affinity of the antibody is directly related to the sensitivity (detection limit) of the immunoassay and to the linearity of its calibration curves. Fab'-nanoAu molecules are approximately 65 kDa and IgG-Fab'-nanoAu complexes are approximately 215 kDa, making separation by gel filtration an attractive alternative to other receptor-ligand binding techniques. Two limitations of the technique include the relatively long period of time it takes for a sample to pass through the column and the resolution of gel filtration. Nevertheless, elemental tagging in combination with ICP-MS has advantages over other means of

detection (for example, UV detector). Non-tagged material such as the BSA, PBS buffer, is completely transparent to an elemental analyzer, allowing low levels of detection and quantitation. This advantage might be used, for example, in quality assurance of biological reagents.

In order to demonstrate this potential two different stock samples of anti-human Fab'-nanoAu were analyzed independently according to the following procedure. 2 mL of 1 : 500 diluted anti-human Fab'-nanoAu was obtained by dilution of stock solutions of Fab'-nanoAu in 1% BSA, PBS buffer plus 1 ppb of Ir. The same iridium spiked buffer was later used as a running buffer. The Fab'-nanoAu was filtered using 1% BSA-blocked 300 kDa Centricon 2 mL filters. The filtered Fab'-nanoAu was run through a sephacryl S200 column. The column was chilled to maintain a temperature of $\sim 8^\circ\text{C}$ in its cooling jacket. Immediately after exit from the column, the eluate was mixed in the ratio 1 : 1 with 10% HCl, 0.1% HF (to improve washout characteristics) plus 1 ppb Tl and run directly into the ICP-MS sample introduction system. The Ir/Tl ratio indicates the mixing proportion and absolute intensities indicate overall signal stability. The iridium signal was used to quantify the gold signal.

In Fig. 2 one can see that, although sufficient for its intended use, additional purification and quantitation of the tagged antibody is required for purposes of elemental analysis. While

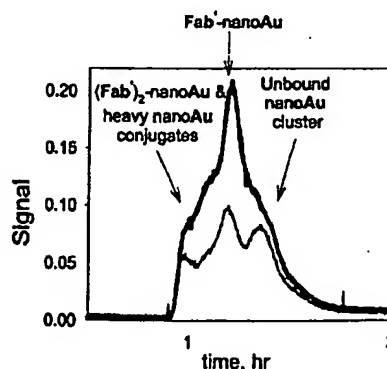


Fig. 2 Results of size exclusion filtration of two different stocks of Fab'-nanoAu. ICP-MS was used as an elemental detector. Signal (the Y axis) represents the $^{197}\text{Au}/(^{191}\text{Ir} + ^{193}\text{Ir})$ ratio.

considered identical by the manufacturer, the samples are different in concentration and in relative amount of contaminants. This experiment obviously demonstrates that the tagged bioactive material specifically designed for the elemental analysis represents some challenge for biotechnology.

ICP-MS linked immunostaining method

ICP-MS can complement the current methods used in proteomics in its never-ending quest for new drugs. Here we demonstrate capabilities of elemental analysis to determine the state of cell line transfection using an immunostaining method with ICP-MS detection. This method was developed based on a widely used fluorescence immunostaining protocol and linking it to elemental detection of metal-tagged antibodies by ICP-MS.

In this experiment, we have used Nanogold antibodies to detect the Smad2 protein that is endogenously present at low levels in C2C12 cells and only expressed at detectable levels in COS cells that have been transfected with an expression vector coding for the Smad2 protein. Both COS and C2C12 cells were grown in 10% FBS on 60 mm plates ($\sim 10^6$ cells). COS cells were transfected at 60% confluency with 5 μ g of pCMV5B-Smad2 (expression vector) using a standard calcium precipitation method. After 48 h, COS cells were fixed to the plate with ice-cold methanol for 10 min at -20°C . C2C12 cells were grown for 48 h (until confluent) and then also fixed with methanol. After drying the plates, the cells were first blocked with 1% BSA/PBS at room temperature for 1 h and then probed with 1 : 1000 rabbit anti-Smad2 antibody for 1 h at room temperature. The cells were washed 4 times for 15 min each time with 1% BSA/PBS and then probed with pre-filtered goat anti-rabbit Fab'-Au. The cells were then washed as before and digested in 1 mL of concentrated HCl. 0.5 mL of the digest was added to 0.5 mL of 10% HCl, 1 ppb Ir and analyzed using the ICP-MS.

As can be seen in Fig. 3, the difference between transfected and control cell lines is clearly observed even in 60 mm plates format ($\sim 10^6$ cells). We are currently applying the method in a 96-well format ($\sim 10^4$ cells) with a low uptake sampling system in order to minimize the sample size and reduce the required number of cells.

Conclusions

ICP-MS brings new features to analysis of biologically active molecules that distinguish it from organic mass spectrometry: relative insensitivity to concomitant organic or metallic species, equivalent sensitivity to all similarly-tagged complexes, inherent ability to provide absolute quantitation and linearity of response. All of these positive features fit the requirements of

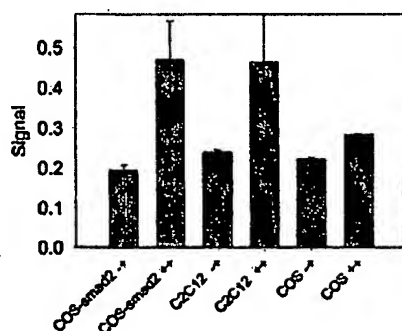


Fig. 3 Results of detection of exogenous Smad2 protein in transfected COS cells and endogenous Smad2 in C2C12 cells in 60 mm plates format. (-): negative control, probed with anti-rabbit Fab'-Au only. (+): probed with both rabbit anti-Smad2 antibody and anti-rabbit Fab'-Au. Signal (the Y axis) represents the $^{197}\text{Au}/(^{191}\text{Ir} + ^{193}\text{Ir})$ ratio.

modern-day biotechnology. In this work the potential of ICP-MS was discussed in the context of several urgent problems of biotechnology including: the detection and quantitation of proteins at the very low level of their natural abundance, control of efficiency of cell transfections and quality control of biologically active materials. The determination of the degree of phosphorylation of proteins in biological samples facilitates opportunity to develop a very sensitive kinase assay.

In addition, ICP-MS determination of an elemental tag attached to a biopolymer offers several advantages over other methods:

- I. the tag is directly analyzed, implying the elimination of at least one step from the conventional immunoassay protocol;
- II. impurities have less impact since they generally do not contain the target elements;
- III. it is reasonable to expect that multiple differently-tagged antibodies can be used to probe one sample for simultaneous determination;
- IV. immediate acidification of the reacted and separated sample allows for long-term storage before analysis and simplifies assay protocols.

References

- 1 K. Inagaki, N. Mikuriya, S. Morita, H. Haraguchi, Y. Nakahara, M. Hattori, T. Kinoshita and H. Saito, *Analyst*, 1999, 125, 197-203.
- 2 J. Wang, R. S. Houk, D. Dreessen and D. R. Wiedner, *J. Biol. Inorg. Chem.*, 1999, 4, 546-553.
- 3 J. L. Neilsen, A. Abildtrup, J. Christensen, P. Watson, A. Cox and C. W. McLeod, *Spectrochim. Acta Part B*, 1998, 53, 339-345.
- 4 J. Wang, R. S. Houk, D. Dreessen and D. R. Wiedner, *J. Am. Chem. Soc.*, 1998, 120, 5793-5799.
- 5 I. Leopold and B. Fricke, *Anal. Biochem.*, 1997, 252, 277-285.
- 6 S. Yoneda and K. T. Suzuki, *Biochem. Biophys. Res. Commun.*, 1997, 231, 7-11.
- 7 I. A. Bergdahl, A. Schutz and A. Grubb, *J. Anal. At. Spectrom.*, 1996, 11, 735-738.
- 8 K. A. High, J. S. Blais, B. A. J. Methven and J. W. McLaren, *Analyst*, 1995, 120, 629-634.
- 9 L. M. Owen, H. M. Crews, R. C. Hutton and A. Walsh, *Analyst*, 1992, 117, 649-655.
- 10 H. Kodama, N. Shimojo and K. T. Suzuki, *Biochem. J.*, 1991, 278(3), 857-862.
- 11 M. Wind, M. Edler, N. Jakubowski, M. Linscheid, H. Wesch and W. D. Lehmann, *Anal. Chem.*, 2001, 73, 29-35.
- 12 P. R. D. Mason, K. Kaspers and M. J. van Bergen, *J. Anal. At. Spectrom.*, 1999, 14, 1067-1074.
- 13 T. Prohaska, C. Latkoczy and G. Stingeder, *J. Anal. At. Spectrom.*, 1999, 14, 1501-1504.
- 14 H. Wildner, *J. Anal. At. Spectrom.*, 1998, 13, 573-578.
- 15 M. Wind, H. Wesch and W. D. Lehmann, *Anal. Chem.*, 2001, 73, 3006-3010.
- 16 D. R. Bandura, V. I. Baranov and S. D. Tanner, *Anal. Chem.*, 2002, 74, 1497-1502.
- 17 E. J. Andreu, J. J. Martin de Llano, I. Moreno and E. Knecht, *J. Histochem. Cytochem.*, 1998, 46, 1199-1201.
- 18 J. J. Martin de Llano, E. J. Andreu and E. Knecht, *Anal. Biochem.*, 1996, 243, 210-217.
- 19 J. J. Martin de Llano, E. J. Andreu, A. Pastor, M. de la Guardia and E. Knecht, *Anal. Chem.*, 2000, 72, 2406-2413.
- 20 V. I. Baranov, D. R. Bandura and S. D. Tanner, *European Winter Conference on Plasma Spectrochemistry, Haffell, Norway 2001*, book of abstracts, p. 85.
- 21 C. Zhang, Z. Zhang, B. Yu, J. Shi and X. R. Zhang, *Anal. Chem.*, 2002, 74, 96-99.
- 22 V. I. Baranov, Z. Quinn, D. R. Bandura and S. D. Tanner, *Anal. Chem.*, 2002, 74, 1629-1636.
- 23 M. Bendayan, *Science*, 2001, 291, 1363-1365.
- 24 J. F. Hainfeld and F. R. Furuya, *J. Histochem. Cytochem.*, 1992, 40, 177-184.
- 25 J. F. Hainfeld and R. D. Powell, *J. Histochem. Cytochem.*, 2000, 48, 471-480.
- 26 J. F. Hainfeld and J. M. Robinson, *J. Histochem. Cytochem.*, 2000, 48, 459-460.
- 27 Nanogold Conjugates, Nanoprobes, <http://www.nanoprobes.com/>.
- 28 J. Cui and J. C. Bystry, *J. Immunol. Methods*, 1992, 147, 13-19.

- 29 R. N. Thomas and C. Y. Guo, *Fresenius' J. Anal. Chem.*, 2001, 369, 477-482.
- 30 DELFIA, Perkin Elmer. Life Sciences, <http://www.perkinelmer.com/>.
- 31 C. Zhang, F. B. Wu, Y. Y. Zhang, X. Wang and X. R. Zhang, *J. Anal. At. Spectrom.*, 2001, 16, 1393-1396.
- 32 J. F. Hainfeld, J. J. Lipka and F. E. Quate, *J. Histochem. Cytochem.*, 1990, 38, 1795-1803.
- 33 J. F. Hainfeld, C. J. Foley, L. E. Maelia and J. J. Lipka, *J. Histochem. Cytochem.*, 1990, 38, 1787-1793.
- 34 R. D. Powell, C. M. Halsey, W. Liu, V. N. Joshi and J. F. Hainfeld, *J. Struct. Biol.*, 1999, 127, 177-184.
- 35 G. L. Wang, J. L. Yuan, B. L. Gong, K. Matsumoto and Z. D. Hu, *Anal. Chim. Acta*, 2001, 448, 165-172.
- 36 S. D. Tanner, V. I. Baranov and U. Vollkopf, *J. Anal. At. Spectrom.*, 2000, 15, 1261-1269.
- 37 V. I. Baranov and S. D. Tanner, *J. Anal. At. Spectrom.*, 1999, 14, 1133-1142.
- 38 S. D. Tanner and V. I. Baranov, *At. Spectrosc.*, 1999, 20, 45-46.
- 39 S. D. Tanner and V. I. Baranov, *J. Am. Soc. Mass Spectrom.*, 1999, 10, 1083-1094.